

Method for characterizing extracellular proteins from the cell wall proteome of the copper tolerant fungus *Phialophora malorum*

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ABSTRACT

Phialophora malorum is a well-known copper tolerant fungus causing soft rot decay of copper-treated wood in-service in ground contact situations worldwide. The aim of the present work was to develop a method that allowed studies of the mechanism(s) behind extracellular copper tolerance by examining the profile of proteins expressed by *P. malorum* in the cell wall/slime (subproteome) environment in mycelia grown in Cu-supplemented media. The study involves development of a novel non-destructive approach using mild extraction, concentration, separation (SDS-PAGE electrophoresis) and characterization (MALDI-TOF MS/MS) of the proteins present. Studies revealed differential protein profiles and expression in Cu-supplemented (0.1% CuSO₄) vs control shake cultures after 10 days growth. Twelve proteins including several hydrolytic (i.e. glucosidases, pectinase, polygalacturonase) enzymes were either unique or over-expressed in Cu-cultures compared to controls. The cell wall location of the proteins was consistent with a role in copper tolerance and detoxification by binding Cu-ions extracellularly. Several of the proteins (e.g. hydrolases, pectinase) are also involved in wood cell wall biomineralization. It is proposed that the concentration of metabolites (organic acids) slowly released in close vicinity of hyphal surfaces by weakly bound enzymes over-expressed through Cu-stress may function to reduce levels of free non-chelated Cu (II) ions entering the cell cytoplasm and therefore provide a first level of defense against copper toxicity.

1. Introduction

Fungi have developed a variety of mechanisms to tolerate toxic levels of heavy metals in their immediate environments (Gadd, 1993; Hall, 2002; Jacobs et al., 2016; Akgul and Akgul, 2018). A number of resistance systems have been advocated including: (i) the active efflux/exclusion/excretion of accumulated metals from cells as described with some yeasts (e.g. Weissman et al., 2000; Ito et al., 2007), (ii) control of metal uptake by complexation at the cell wall with extracellular polymeric slime materials (EPS) (Daniel and Nilsson, 1989; Yahaya et al., 2009) or chelation/precipitation by soluble secreted metabolites (El-Meleigy, 1992) such as oxalic acid (Jarosz-Wilkolazka and Graz, 2006; Clausen et al., 2000; Clausen and Green, 2003; Green and Clausen, 2003), (iii) detoxification of accumulated metal ions by intracellular complexation/immobilization by binding to intracellular protein chelators like metallothioneins (e.g. Schwartz et al., 2013), and (iv) metal

sequestration in the cellular periplasm or vacuoles (Pearce and Sherman, 1999). Typically, these mechanisms may function individually or in combination to prevent a fungus from heavy metal toxicity stress, their purpose being to reduce damage to biogenic macromolecules sensitive to oxidative species derived from reactive metal ions.

Fungi with an ability to degrade waterborne preservative treated wood (i.e. wood treated with metals like chromated-copper-arsenate (CCA), or copper treatments; Mantanis et al., 2014; Humar and Thaler, 2017) are recognized to have metal tolerance or possess mechanisms to prevent metal stress and death. Their ability to degrade metal-treated wood and at the same time tolerate heavy metals has generally been ascribed as giving such fungi a competitive advantage although this hypothesis has been questioned recently for soft rot fungi (Karunasekera et al., 2017). Previous studies on different *Phialophora* spp. causing soft rot on copper-treated wood have shown certain species

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(e.g. *P. malorum* and *P. mutabilis*) to grow *in-vitro* on media containing high copper concentrations (i.e. as CuSO₄ or micronized copper) (Daniel and Nilsson, 1988; Karunasekera and Daniel, 2013) indicating strong Cu-tolerance. Earlier, electron microscopy and X-ray micro-analytical studies have shown *P. mutabilis* to bind copper both intracellularly to cellular components and extracellularly to cell wall/slime (subproteome) materials suggesting that a variety of mechanisms may be involved (Daniel and Nilsson, 1989). *Phialophora malorum* and *P. mutabilis* when grown *in-vitro* on CuSO₄ supplemented media cause green discoloration of the culture fluid and bind copper to mycelia suggesting employment of an extracellular mechanism (i.e. external to cell membrane) for copper reduction possibly involving proteins or extracellular chelators (Daniel and Nilsson, 1988; Karunasekera et al., 2019). In a previous study, protein expression by *P. malorum* mycelia grown in CuSO₄ media were examined using a global proteomic approach that showed a number of characteristic proteins up- or down-regulated during growth in copper supplemented media (Karunasekera et al., 2019). The global approach however, involved analysis of the entire *P. malorum* proteome and while some information can be retrieved indirectly from the dataset on metabolic functions and likely cellular distribution, considerably less information is available for specific cellular structures and regions. For *P. malorum* this was complicated further as the genome is currently unknown (Karunasekera et al., 2019).

The aim of the present study was to develop a specific method to examine the profile of extracellular proteins (i.e. proteins weakly bound to the fungal cell wall/slime) differentially expressed in the cell wall proteome in Cu-supplemented vs control *in-vitro* liquid cultures. Work included development of a non-destructive approach involving mild protein extraction, concentration, separation (via electrophoresis) and identification via sequencing of polypeptides (MALDI-TOF MS/MS). *Phialophora* spp. are well known for their ability to degrade lignified wood cell walls through cavity (Type I; characteristic cavities in wood cell walls) and erosion (Type II; surface erosion of wood cell walls) decay (Daniel and Nilsson, 1989, 1998; Daniel, 2016) and are known to produce both extracellular cellulases and hemicellulases (Nilsson, 1974; Berg, 1978; Duncan et al., 2006) *in-vitro* on agarose/agar plates. *Phialophora* are a group of cosmopolitan anamorph fungi with worldwide distribution having been isolated from a wide range of Cu-treated wood in-service in ground contact situations (Daniel and Nilsson, 1988; Daniel, 2016).

2. Materials and methods

2.1. Fungal cultures

Phialophora malorum (strain 211-C-15-1) (M. N. Kidd & A. Beaumont) McCulloch (syn = *Cadophora malorum*; Harrington and McNew, 2003) was obtained from the culture collection maintained at the Department of Biomaterials and Technology/Wood Science where it is routinely grown on 2.5% w/v malt extract agar (MEA) at 20 °C. The strain was previously isolated from 2% w/v CCA treated poles (Henningsson and Nilsson, 1976). The fungus was grown in 500-mL shaken flasks (VKS Edmund Buhler horizontal rotary shaker; 80 rpm) containing Abram's basic salt medium (KH₂PO₄, 0.1% w/v; MgSO₄·7H₂O, 0.1% w/v) amended with glucose (2% w/v), peptone from animal tissue (Sigma 5905) (0.05% w/v) and glutamic acid (0.05% w/v) at room temperature. Details on culturing have been described previously (Karunasekera and Daniel, 2013). Under these conditions the fungus forms mycelia pellets. For Cu-cultures, the medium was supplemented with 0.1% w/v CuSO₄ (Sigma). Preliminary analysis of *P. malorum* cultures were also conducted with both young (i.e. 4 days) and old cultures (i.e. 6 weeks that was used in previous experiments) to determine via electrophoresis the nature of the protein profiles. The 6 week old cultures produced very complicated and dense electrophoresis profiles where it was difficult to distinguish and

characterise discrete protein bands associated with copper, while the 4 day old cultures were too early to see an effect of copper. Thus after several trials, a 10 day culture period was selected which was also within the logarithmic growth phase. A rather low copper concentration was used (i.e. 0.1% CuSO₄) compared with what *P. malorum* has been shown to tolerate in *in-vitro* liquid cultures and on Cu-agar (Daniel and Nilsson, 1988, 1989; 1998; Karunasekera and Daniel, 2013) as this concentration was shown previously to provide a stimulatory effect reflected by an increase in mycelial dry wt compared with control cultures (Karunasekera and Daniel, 2013).

2.2. Extraction of fungal mycelia

Numerous trials with different extraction buffers were examined. However, the buffer providing optimal protein extraction contained 200 mM CaCl₂, 10 mM EDTA, 10% v/v glycerol and protease inhibitors (2 mM Pefabloc and 10 µg/ml each of aprotinin, leupeptin, pepstatin, antipain and soybean trypsin inhibitor) in 50 mM BisTris-HCl, pH 4.5 (after dissolution of CaCl₂, glycerol and EDTA in 50 mM BisTris-HCl pH 6.0, the pH dropped to ca 3.5; pH was then adjusted to 4.5).

Mycelia from 10-day-old shake cultures grown in 0.1% w/v CuSO₄ and control cultures without copper were harvested on sintered glass (no. 2) funnel, washed 4x with the same volume of distilled water (ca. 20 mL), drained of excess water and frozen at -20 °C. Two g wet wt. mycelia for each sample was then extracted for 2 h at 4 °C into 20 ml of the extraction buffer in 50-ml plastic vials fixed on a vertical rotator. Extracts were then separated from mycelia on a sintered glass funnel under mild vacuum and prior to ultrafiltration (UF) concentration, using a 0.45 µm syringe filter holder (Minisart NML, Sartorius). Studies revealed no difference in protein profiles using either fresh or previously frozen (-20 °C) mycelia.

Light- and fluorescence microscopy observations (not shown) of mycelia after staining with DAPI (4'-6-diamino-2-phenylindole, Sigma Aldrich) (Karunasekera and Daniel, 2013) following extraction showed hyphae with cellular content and viable nuclei proving the extraction process was mild and non-destructive (i.e. the cell membrane remained intact).

2.3. Experimental set-up for extract concentration of copper grown and control cultures

Extracts of *P. malorum* mycelia grown in 0.1% w/v CuSO₄ was concentrated on PLGC (Millipore 04310) NMWL 10 kDa regenerated cellulose UF membranes, ∅ 44.5 mm (Millipore) to apparent 'dryness', followed by addition of 0.6 ml of 100 mM NaCl in 50 mM Tris-HCl pH 8 and stirred without pressure for 10 min. The concentrated sample was then transferred to 2-mL Eppendorf tubes and centrifuged for 10 min at 15 000 g to remove any precipitates. Soluble proteins in the supernatant made up to 750 µL were precipitated for 2 h on ice using 100% TCA (132.4 µL) added slowly under vigorous mixing (resulting 15% TCA). Precipitated proteins were subsequently pelleted by centrifugation at 15 000 g for 15 min. The pellet was then washed with ice-cold acetone (2 x 200 µL), vortexed and spun down at 10 000 g for 10 min (MIKRO 22 R table centrifuge). Extracts of 2 g wet wt. mycelia from control flasks without copper were prepared, concentrated and precipitated as described for copper cultures.

2.4. SDS-PAGE

Extracts from copper- and control cultures were dissolved in 100 µL SDS sample buffer and heated for 5 min at 95 °C with vortex mixing at 3 and 5 min. Proteins were fractionated using a 10% gel at loadings of 5, 10 and 20 µL per well and detected using silver (Fig. 1). A range of protein markers (25–110 kDa) was included as standards. SDS-PAGE gels were run 5x with similar results from different experiments.

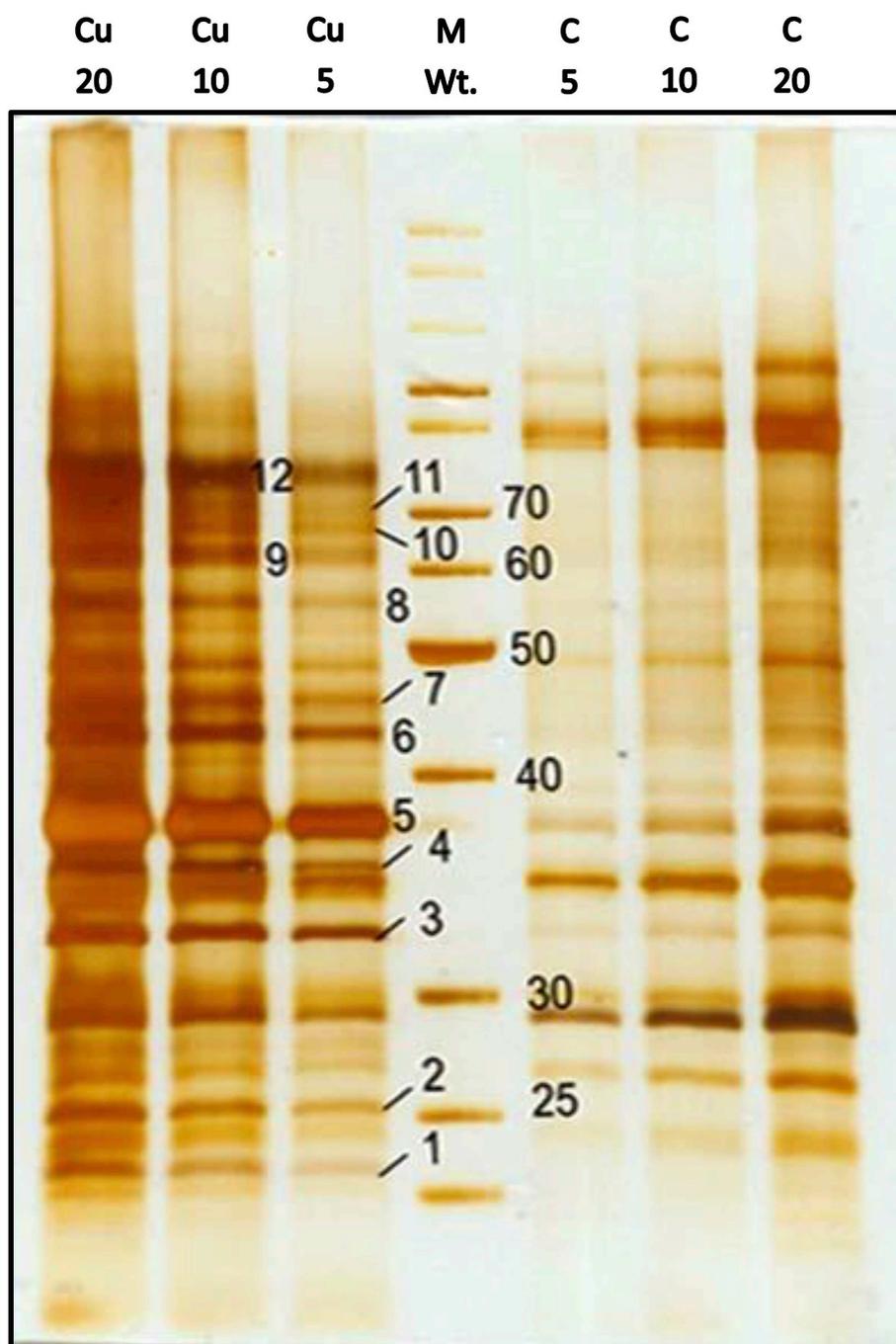


Fig. 1. SDS-PAGE of proteins extracted from the extracellular subproteome of *P. malorum* (10 day old cultures). From left to right, loading scheme was: (Cu) 20, (Cu) 10, (Cu) 5 μ L of *P. malorum* Cu-sample extract and (c) 5, (c) 10, (c) 20 μ L control extracts. Molecular weight standards in kDa are shown in the central lane. Numbers 1–12 represent bands selected for subsequent MALDI-TOF MS/MS analysis. Comparison of the profiles shows that a number of bands present in the Cu-extract are either missing or very weak in the control extracts removed after the same growth period.

2.5. Enzymatic digestion for MALDI-TOF MS/MS

Silver-stained protein bands excised from gels, were cut into small pieces and washed with a freshly prepared solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (mixed in 1:1 ratio). After complete destaining, the proteins were reduced with 30 mM TCEP in 100 mM Tris-HCl (pH 8.0) at 65 °C for 30 min and alkylated by 30 mM iodacetamide in 100 mM Tris-HCl (pH 8.0) for 60 min in the dark. The gel was washed further with water, shrunk by dehydration in acetonitrile (MeCN) and re-swelled in water. The supernatant was removed and the gel partly dried in a SpeedVac concentrator. The gel pieces were then incubated overnight at 37 °C in a cleavage buffer containing

25 mM 4-ethylmorpholine acetate, 5% MeCN and trypsin (100 ng; Promega). Tryptic digestion was performed in a mixture of H₂¹⁶O and isotopic H₂¹⁸O (1:1) leading to incorporation of ¹⁶OH as well as ¹⁸OH at the C-terminus of each newly formed peptide. In MS/MS analysis of such ¹⁶O/¹⁸O labelled peptides, N-terminal b-ions appeared with their normal isotopic pattern, whereas C-terminal y-ions had a typical profile of doublets differing by 2 Da. This feature clearly distinguished b- and y-ions and thus facilitated the assignment of peptide sequences. The resulting peptides were extracted to 40% in MeCN/0.1% TFA. An aqueous 50% MeCN/0.1% TFA solution of α -cyano-4-hydroxycinnamic acid (5 mg/ml; Sigma) was used as a MALDI matrix. One μ l of the peptide mixture was deposited on the MALDI plate, allowed to air-dry

at room temperature and overlaid with 0.4 μL of the matrix.

2.6. MALDI mass spectrometry and protein identification

Mass spectra were measured on an Ultraflex III MALDI-TOF MS/MS instrument (Bruker Daltonics, Bremen, Germany) in the mass range of 700–4000 Da and calibrated internally using the monoisotopic $[\text{M} + \text{H}]^+$ ions of trypsin auto proteolytic fragments (842.5 and 2211.1 Da). The amino acid sequences manually derived from the acquired MS/MS spectra were searched against NCBI nr_2016_06 database using the MS-BLAST program (<http://genetics.bwh.harvard.edu/msblast/>) developed for short sequence tags extracted from the MS data. Because the genome of *P. malorum* is unknown, polypeptides were identified on the basis of high sequence homology with related fungal species (phylogenetic, habitat and ability to degrade natural polymers). To confirm the identity of the peptides, their exact masses were measured in MALDI mode using a Solarix XR™ FT-ICR mass spectrometer (Bruker Daltonics) with mass accuracy below 2 ppm.

3. Results

3.1. General culturing

After 10 days growth the culture fluid of the Cu-supplemented cultures had a slight green-blue appearance, as did the filtered mycelia in comparison to controls consistent with previous studies (Daniel and Nilsson, 1988; Karunasekera et al., 2019). During protein extraction, the distinctly greenish-blue mycelia changed colour becoming more creamy-white similar to the control samples suggesting loss of Cu (II) ions from the fungal cell wall and extracellular slime materials.

3.2. SDS-PAGE

Fig. 1 shows typical protein profiles obtained with different loadings (5–20 μL) of extracted extracellular proteins isolated by mild extraction of *P. malorum* mycelia after 10 days growth. Comparison of the protein profiles shows several bands present in the Cu-extracts (i.e. bands marked 1–12 between ca 25 and 110 kDa, Fig. 1) as absent or very weak in the control extracts taken after the same growth period suggesting differential expression of unique proteins or strong up-regulation of existing proteins.

3.3. MALDI-TOF MS/MS protein identification

Silver-stained protein bands were in-gel trypsin-digested and the peptides identified using MALDI-TOF MS/MS (Table 1). Identity of the proteins was revealed based on the high sequence homology with related species from similar phylogenetic groups and soil habitat or capability for decaying natural polymers (e.g. cellulose). Analysis of the 12 bands showed a variety of protein sequences that could be ascribed to a number of enzymes including: peptidases, proteases, pectinase/polygalacturonase and glucosidases as well as hypothetical proteins (Tables 1 and 2). In particular, the hydrolases glucosidases, peptidases, pectinase/polygalacturonase were dominating (Fig. 1; Tables 1 and 2).

4. Discussion

Considerable research to clarify physiological and molecular mechanisms underlying metal tolerance by a variety of microorganisms particularly copper in fungi has been conducted (Gadd, 1993, 2007; Tang et al., 2013; Akgul and Akgul, 2018). The most effective mechanisms would be those preventing microbial cells from excessive intracellular copper uptake. With some yeasts, this has been shown through strong down-regulation of the expression of *CTR1* and *FRE1* genes responsible for copper intracellular transport in its reduced Cu (I) form and up-regulation of a Cu (I) oxidase *FET3* gene to reduce copper

uptake by means of *CTR1p*. Other complementary mechanisms are believed to contribute to the defense strategy such as copper adsorption on the cell wall/extracellular polymeric slime (EPS) (i.e. subproteome) (Yahaya et al., 2009) and by extracellular metal complexation/chelation (El-Meleigy, 1992). In this study, we have focused on the latter possibility by assuming that the *P. malorum* cell wall components expressed under heavy copper load may represent a primary barrier against free Cu (II) ion diffusion (i.e. from CuSO_4) towards the cell membrane. The hydrolytic enzymes we found (i.e. hydrolases/peptidases/glucosidases) weakly bound to the cell wall/slime and differentially over-expressed during exposure to high copper concentrations all represent a source of soluble, heavy metal complexing metabolites that can be accumulated close to the cell surface. The substrates for these enzymes are presumably the wood cell wall carbohydrate polymers and proteins of the copper-treated wood during soft rot erosion (i.e. from wood cell lumen) and cavity formation inside wood cell walls (Daniel and Nilsson, 1989; Daniel, 2016).

It is well documented that copper forms soluble complexes with amino acids (hydroxyl), carboxylic acids and carbohydrates (Beck, 2009). The mechanism of heavy metal detoxification by chelation into less toxic species with suppressed bioavailability has also been discussed. Thus, increased secretion of metal-binding metabolites such as citrate, malate and histidine could also reduce uptake of toxic free metal ions into plant cells (Rausser, 1999). For example, cells of the diatom *Cylindrotheca fusiformis* exposed to toxic Cu concentrations displayed higher carbohydrate extracellular production that paralleled the increase in Cu concentrations (Pistocchi et al., 1997). Complexation of cupric ion has also been shown to have a profound effect on ion toxicity towards bacteria. With studies on the Cu-sensitive and Cu-tolerant strains of phytopathogenic bacterium *Pseudomonas syringae*, Cu (II) toxicity was substantially reduced in the presence of a variety of organic compounds (i.e. acids, amino acids and sugars), indicating that not only strong chelating agents but also weak and moderate copper ligands can effectively antagonize copper toxicity. In this respect, the discoloration of the culture media in *P. malorum* cultures is suggestive that a similar process may be in operation. Interestingly, the complexed forms of copper were not toxic to strains of *P. syringae* (Zevenhuizen et al., 1979; Menkissoglu and Lindow, 1991; Azenha et al., 1995). Obviously, there is also a competition between soluble ligands (chelators) in solution and polymeric ligands on the hyphal cell surface, affecting the concentration of free Cu (II) (Teresa et al., 1997). In copper tolerant wood-rot fungi, a role of metal complexation/immobilization in insoluble crystals has been mostly attributed to oxalic acid secreted in elevated levels during growth under Cu-stress (Clausen et al., 2000; Clausen and Green, 2003; Green and Clausen, 2005; Jarosz-Wilkolazka and Graz, 2006; Köse and Kartal, 2010; Ohno et al., 2015; Lenz et al., 2017; Akgul and Akgul, 2018). Eight genes of the brown rot fungus *Fibroporia radiculosa*, up-regulated during growth of this fungus on wood treated with a copper-based preservative, had putative functions related to operation of an oxalate system (Tang et al., 2013). In contrast, *P. malorum* has not been shown to produce excess cell wall oxalate crystals attached to hyphal cell walls in copper supplemented cultures and thus the involvement of a similar oxalic acid system is unlikely (Karunasekera et al., 2019).

Our finding for the presence of pectinase/polygalacturonase proteins is also consistent with previous studies. For example, a possible role of pectinase/polygalacturonase in fungal copper tolerance mechanisms was documented in the transcriptomic study on *F. radiculosa* grown on copper-treated wood. Among several differentially regulated genes, two genes -viz 1513, GH28 polygalacturonase, and 2569, GH43 endo-1,5- α -L-arabinanase, up-regulated at early stages of wood attack had annotations for pectin degradation (Tang et al., 2013). Pectin, an acidic polysaccharide which is largely present in plant cell walls, is a frequent target of the primary attack of wood rotting fungi. The most abundant component in pectin (ca 70%) is polygalacturonic acid, a linear polymer of 1,4- α -linked partially methyl-esterified d-

Table 1

MALDI-TOF mass spectrometric identification of *P. malorum* extracellular proteins over-expressed in Cu-cultures. Table includes band number, protein name, NCBI database number, fungus species, theoretical protein molecular weight, and sequence, experimental and theoretical protonated MW and mass error of identified peptides.

Band	Protein name	NCBI No.	Fungal organism	Protein MW theor. [kDa]	Peptide sequence identified by MALDI-TOF MS/MS analysis	Peptide MH + exp. [Da] [§]	Peptide MH + theor. [Da]	Error [ppm]
01	-							
02	Serin endopeptidase	406859900	<i>Marssonina brunnea</i>	26	YVSVPVVSR IVGGSAAVAGQFPYQVALLR	1005.5729 2017.1240	1005.5728 2017.1229	0.1 0.5
03	Putative aspartic protease	82173725	<i>Trichoderma harzianum</i>	40	YTGTLTYFSR FLENYYSVYDTTNSR YYAWINDGGSSGVNTIIGQK	1208.5943 1871.8455 2143.0483	1208.5946 1871.8446 2143.0455	0.2 0.5 1.3
04	Hypothetical protein MBM_06090	406863030	<i>Marssonina brunnea</i>	29	GVVYLQGVLR IFLPTTPSEPSLPSVTPLPISR	1103.6573 2349.3089	1103.6572 2349.3064	0.1 1.1
05	Glycoside hydrolase family 28 protein	451847121	<i>Bipolaris sorokiniana</i>	37	FFYAHSLTSSITGLNFK QTPQFFYAHSLTSSITGLNFK	2034.0336 2488.3216	2034.0331 2488.3235	0.2 0.8
06	Acid protease	512197988	<i>Glarea lozoyensis</i>	43	NQIFAEAATEEPGLAFAFGR IKNQIFAEAATEEPGLAFAFGR	2068.0141 2309.1943	2068.0134 2309.1925	0.3 0.8
07	Glycoside hydrolase family 31 protein	972541695	<i>Phialocephala scopiformis</i>	104 [#]	WATLGAFTSYFR STFSGAGSHVGHWLGDNVSSWLGYSR SPGDDVALIDLPHYQGGPDVPQR [*]	1419.7057 2677.2549 2858.5610	1419.7056 2677.2542 n.a.	0.1 0.3 n.a.
	Pectin lyase A	115401252	<i>Aspergillus terreus</i>	40	GNYYHTSGR	1167.5542	1167.5542	0
08	-							
09	Glycoside hydrolase family 31 protein	972541695	<i>Phialocephala scopiformis</i>	104 ^{&}	YTASPFSSITR IQDAADLVYQLPASVLP IQDAADLVYQLPASVLP QIVMVDPAVAYQPPYPPYQR	1376.6848 1969.0769 2194.2237 2235.1284	1376.6845 1969.0753 2194.2230 2235.1267	0.2 0.8 0.3 0.8
10	Tripeptidyl-peptidase 1 precursor	406863634	<i>Marssonina brunnea</i>	81 [#]	VCNGFAQLGAR ADGTIPWPYPGIHEAR NQGFNNFLGELPIRPDTK	1192.5897 1779.8824 2173.1406	1192.5892 1779.8813 2173.1400	0.4 0.6 0.3
11	Tripeptidyl-peptidase 1 precursor	406863634	<i>Marssonina brunnea</i>	81 [#]	GLFNPEGR VCNGFAQLGAR FLASFPPSCPYPVAVGATQGFEPETSAAR	889.4521 1192.5888 3058.4627	889.4526 1192.5892 3058.4615	0.6 0.3 0.4
12	Hypothetical protein MBM_07560	406861275	<i>Marssonina brunnea</i>	73	SGLGLFNPVLYR VISASYGQAEADLPAPYVR	1335.7432 2007.0192	1335.7420 2007.0182	0.9 0.5
	Subtilisin-like protein	512200580	<i>Glarea lozoyensis</i>	72	NQCGLYKPTR VISVSYGVSEYDAPLSYTK AHDPLPYYVANDAATNIGANGGLYNR	1236.6150 2078.0325 2804.3409	1236.6154 2078.0328 2804.3387	0.3 0.1 0.8

[#] Identified peptides match to the C-terminal part of the protein.

[&] Identified peptides match to the N-terminal part of the protein.

^{*} Only partial peptide sequence was identified.

[§] Measured on MALDI-FTICR mass spectrometer.

n.a.; not applied.

galacturonate units. Free carboxylic groups of pectins are able to bind strongly polyvalent metal cations including Cu (II) (Cataldo et al., 2012). Accordingly, a substantial portion of the Cu that accumulated into cell walls of the Cu-treated *Lygodium japonicum* (fern) and *Scopelophila cataractae* (moss) cells was tightly bound to the homogalacturonan of cell wall pectin (Konno et al., 2005; Konno and Nakashima, 2010).

Possible modes of complexation of Cu (II) cations by D-galacturonic

acid (GalA) in solution or in solid state have also been widely investigated and several ways of coordination binding have been suggested (Debongnie et al., 1987; Synytsya et al., 2004; Shestavin et al., 2003). Due to the COOH group, the complex-forming ability of the sugar acid is known to be highly enhanced relative to neutral sugars, even in acidic solution (pH > 3) (Shestavin et al., 2003; Gyurcsik and Nagy, 2000). Stronger coordination bonds were formed with monomeric galacturonic acid compared to its polymeric form in pectin

Table 2

Extracted extracellular proteins, putative metabolic activities and fungal specie assignment.

Protein name	Putative activity (Metabolic process)	Phylogenetic assignment of peptides (Fungal species)
1.	No protein identified	
2. Serin endopeptidase	Trypsin-like serine protease	<i>Marssonina brunnea</i>
3. Putative aspartic protease	Pepsin-like aspartic protease	<i>Trichoderma harzianum</i>
4. Hypothetical protein MBM_06090		<i>Marssonina brunnea</i>
5. Glycoside hydrolase family 28 protein	Polygalacturonase	<i>Bipolaris sorokiniana</i>
6. Acid protease	Pepsin-like aspartic protease	<i>Glarea lozoyensis</i>
7. Glycoside hydrolase family 31 protein	Alpha/beta glucosidase	<i>Phialocephala scopiformis</i>
Pectin lyase A	Pectinase	<i>Aspergillus terreus</i>
8.	No protein identified	
9. Glycoside hydrolase family 31 protein	Alpha/beta glucosidase	<i>Phialocephala scopiformis</i>
10. Tripeptidyl-peptidase 1 precursor	Peptidase S53	<i>Marssonina brunnea</i>
11. Tripeptidyl-peptidase 1 precursor	Peptidase S53	<i>Marssonina brunnea</i>
12. Hypothetical protein MBM_07560	Peptidase S53	<i>Marssonina brunnea</i>
Subtilisin-like protein	Peptidase S53	<i>Glarea lozoyensis</i>

(Debondonie et al., 1987). Thus, pectinase activity elicited at the hyphal surface during soft rot attack of Cu-treated wood may provide *P. malorum* not only with a nutrient sugar but also with an efficient copper chelator. Interactions between metal ions and other carbohydrates have also been shown to play a role in toxic metal metabolism. For example, two components demonstrated to bind Ni^{2+} in human kidney were identified as small acidic oligosaccharides and a ~3.5 kDa acidic peptide (Predki et al., 1992). Synthesis of sugar acids (formic, malic, oxalic) in liquid media by the white rot fungus *Trametes versicolor* in the presence of CuHDO active ingredients copper oxide, boric acid and CuHDO [bis-(N-cyclohexyl-diazoniumdioxo) copper] have also been reported (Betlej and Graz, 2006).

Over-expression of glucosidases (Fig. 1; Table 2) identified in our *P. malorum* cell wall proteomic study may also contribute to *P. malorum* copper resistance in a similar way as pectinase, although, probably to a lesser extent due to weaker chelation. In this context, neutral reducing monosaccharides were also recognized in solution to undergo extensive coordination with polyvalent metal cations such as Cu (II) (González et al., 2010; Pal and Paul, 2008). While there is vast literature focused on the structural chemistry of stable carbohydrate-metal ion coordination complexes formed under strongly binding conditions in alkaline media, the chemistry of metal-non-deprotonated sugar complexes is still a challenging field. This is due to the relatively low stability of the complexes in neutral or acidic aqueous solutions, and the fact that in solutions of sugars, the species are in anomeric and conformational equilibrium, and the isomers interact in different ways with metal ions. There is, nevertheless, strong evidence that these ligands (sugars in pyranose or furanose form) produce 1:1 complexes with metal cations in hydrophilic solvents where coordination of oxygen atoms of non-deprotonated OH groups takes place (Gyurcsik and Nagy, 2000). The coordination behavior between metal and sugar OH groups is therefore important not only to understand with respect to the physiological role of metal ions (Allscher et al., 2008) but also complexation as is the case with *P. malorum*. Strong metal chelating power of amino acids and peptides, produced through the Cu-affected *P. malorum* peptidases/proteases/hydrolases we have detected is also well known (Ytreberg et al., 2011).

In summary, the concentration of metabolites released in close vicinity of the hyphal surface (i.e. during soft rot erosion and cavity formation) by weakly bound hydrolytic enzymes overexpressed in response to copper stress may be high enough to effectively reduce the level of free/non-chelated Cu (II) ions available to the native 'biotic' ligand such as Fre1p involved in copper intracellular transport. The resulting reduced intracellular copper accumulation in combination with presumed internal mechanisms of the metal sequestration, extrusion, and detoxification may therefore confer high resistance to the environmental copper exposure to/on *P. malorum* hyphae.

5. Conclusions

A novel approach has been developed for characterizing extracellular proteins/peptides present in the cell wall/extracellular slime subproteome (i.e. lying outside the cell membrane) of the Cu-tolerant soft rot fungus *P. malorum*. Twelve proteins/peptide fractions were either unique or over expressed in copper cultures. The subproteome location of the proteins was consistent with a role in copper tolerance/Cu-detoxification by binding Cu ions extracellularly. Several of the proteins isolated (e.g. hydrolases) are also implicated in wood cell wall attack and likely operate with organic acids to bind excess Cu ions released during decay. The subproteome approach developed provides a novel way to extract and profile extracellular proteins from fungal hyphae that may be applied in other studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ibiod.2019.104769>.

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